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INDUCTION OF ANTIBODIES AGAINST GD3 GANGLIOSIDE IN MELANOMA PATIENTS BY VACCINATION WITH GD3-LACTONE-KLH CONJUGATE PLUS IMMUNOLOGICAL ADJUVANT QS-21

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The gangliosides GD3, GD2 and GM2 are expressed on the cell surface of malignant melanomas, GD3 being the most abundant. We have shown that immunization of melanoma patients with GM2 adherent to Bacillus Calmette-Guerin (GM2/BCG) induced an IgM antibody response. Vaccines containing GM2-keyhole limpet hemocyanin (KLH) conjugate and the immunological adjuvant QS-21 induced a higher titer IgM response and consistent IgG antibodies. Patients with antibodies against GM2 survived longer than patients without antibody. On the other hand, our previous trials with GD3/BCG, GD3 derivatives including GD3-lactone (GD3-L)/BCG failed to induce antibodies against GD3. In our continuing efforts to induce antibody against GD3, we have immunized groups of 6 melanoma patients with GD3-KLH or GD3-L-KLH conjugates containing 30 μg of ganglioside plus 100 μg of QS-21 at 0, 1, 2, 3, 7 and 19 weeks. Prior to vaccination, no serological reactivity against GD3 or GD3-L was detected. After immunization, IgM and IgG antibodies were detected against both GD3 and GD3-L in the GD3-L group exclusively. The GD3-L-KLH vaccine induced IgM titers against GD3-L of 1:40–1/1,280 in all patients and IgG titers of 1/160–1/1,280 in 4 patients. These antibodies also strongly cross-reacted with GD3. ELISA reactivity was confirmed by immune thin-layer chromatography on GD3 and melanoma extracts. Sera obtained from 4 of these 6 patients showed cell surface reactivity by FACS and from 2 showed strong cell surface reactivity by immune adherence (IA) assay and complement lysis against the GD3 positive cell line SK-Mel-28. *Int. J. Cancer* 85: 659–666, 2000.
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Gangliosides are sialic acid containing glycosphingolipids composed of a carbohydrate moiety attached to ceramide. Gangliosides GM2, GD2 and GD3 are expressed on the cell surface of human malignant melanomas and other tumors of neuroectodermal origin. These antigens have been demonstrated to be susceptible targets for treatment with monoclonal antibodies (MAbs) and vaccines (Jurcic *et al.*, 1997; Livingston, 1995; Ragupathi, 1996; Scheinberg and Chapman, 1995). We and others have shown that the presence of antibodies against GM2 (either naturally or vaccine induced) has been associated with an unexpectedly favorable disease-free and overall survival. We demonstrated that the optimal way to immunize against GM2 is by vaccinating with GM2 covalently conjugated to keyhole limpet hemocyanin (GM2-KLH) plus immunological adjuvant QS-21 (Helling *et al.*, 1995). However, GD3 is the dominant melanoma ganglioside and we have been unable to induce antibodies against GD3 in melanoma patients by active immunization with GD3-expressing melanoma cells or purified GD3 or GD3 congeners such as GD3-lactone plus Bacillus Calmette-Guerin (GD3-L/BCG) (Ritter *et al.*, 1991). However, GD3-L has been reported to be a more effective immunogen than GM3 (Nores *et al.*, 1987), presumably due to the increased molecular rigidity resulting from lactone ring formation, and the KLH conjugate vaccine is more immunogenic than the previous BCG vaccine. Consequently, we have tested the immunogenicity in melanoma patients of GD3-KLH plus QS-21 and GD3-L-KLH plus QS-21 vaccines and report the successful induction of antibodies against purified GD3 and melanoma cells expressing GD3 in the majority of patients.

MATERIAL AND METHODS

Material

GD3 extracted from bovine buttermilk was received from Matreya (Pleasant Gap, PA). GM1, GM2, GM3 and GD2 extracted from bovine brain, BSA, sodium cyanoborohydride, 4-chloro-1-naphtol and *p*-nitrophenyl phosphate were purchased from Sigma (St. Louis, MO). QS-21 (Kensil *et al.*, 1991) was obtained from Aquila (Framingham, MA). Clinical grade KLH was obtained from Perlmmune (Rockville, MD). Goat anti-human IgG and IgM conjugated with alkaline phosphatase obtained from Kierkegaard and Perry (Gaithersburg, MD) were used for ELISA. Goat anti-human IgM or IgG labeled with fluorescein isothiocyanate (FITC) were obtained from Southern Biotechnology Associates (Birmingham, AL) and used in a fluorescence-activated cell sorter (FACS). Horseradish peroxidase-conjugated goat anti-human IgM and IgG purchased from TAGO (Burlingame, CA) was used for dot-blot immune staining and immune thin-layer chromatography (ITLC). Rabbit anti-mouse immunoglobulins conjugated with horseradish peroxidase were obtained from Zymed (San Francisco, CA) and used for ITLC with mouse control MAb R24 against GD3 (Pukel *et al.*, 1982). High performance thin-layer chromatography (HPTLC) silica gel plates were obtained from Merck (Darmstadt, Germany).

Vaccine preparation

GD3-KLH conjugate was prepared as described previously (Fig. 1a) (Helling *et al.*, 1994). The principle involved in the conjugation procedure is cleavage of the double bond of ceramide by ozone, generation of an aldehyde group and conjugation to ε-amino groups on lysine of KLH by reductive amination. The GD3-KLH conjugate was prepared in 3 batches. The amount used for conjugation, the percent recovery and the GD3/KLH epitope ratio for the GD3-L-KLH vaccine are summarized in Table I. More than 23% of GD3 in the reaction mixture was conjugated with KLH. The GD3/KLH epitope ratio for the combined preparation was 1,049.

GD3-L-KLH vaccine. Because of the unstable nature of GD3-L, we firstly prepared the GD3-KLH conjugate then converted it to GD3-L-KLH by acid treatment (Fig. 1b) and lyophilized immediately. Briefly, equal volumes of GD3-KLH and glacial acetic acid (v/v) were mixed in a sterile glass tube. To monitor GD3-L conversion, the conversion of free GD3, a small portion of which had not been removed completely from the conjugation reaction, was determined by TLC. After 4 hr at 37°C with gentle shaking, when about

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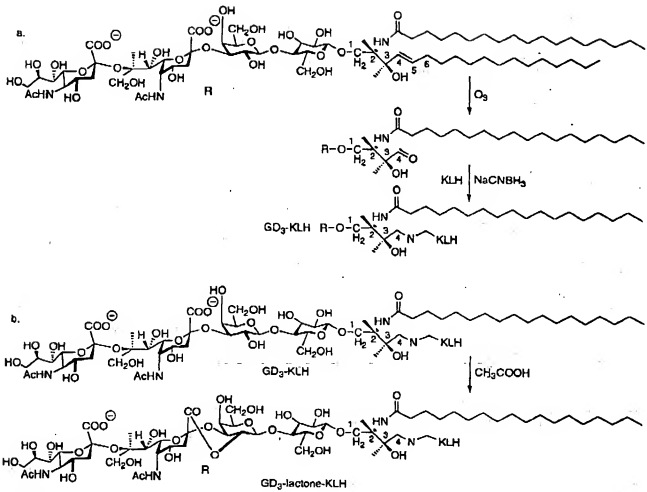

FIGURE 1 – (*a*) Synthesis of GD3-KLH conjugate after ozone cleavage and reductive amination. (*b*) Conversion of GD3-KLH conjugate to GD3-L-KLH conjugate.

TABLE I- PREPARATION AND ANALYSIS OF THE GD3-KLH CONJUGATE USED TO PREPARE THE GD3-KLH AND GD3-L-KLH VACCINES

Batch number[1]	Amount used for conjugation (mg)		Ratio by weight GD3:KLH	The amount of GD3 and KLH in conjugate (mg)		Recovered (%)		Epitope ratio of conjugate GD3/KLH
	GD3	KLH		GD3	KLH	GD3	KLH	
1	5.0	7.5	1:1.5	0.90	5.04	18.0	67.2	1,076.0
2	3.0	5.0	1:1.6	0.78	4.08	26.0	81.6	1,118.2
3	5.0	6.0	1:1.2	0.99	6.00	19.8	100	952.5
Combined	13.0	18.5	1:1.4	2.67	15.12	20.5	81.7	1,048.9

[1]All 3 batches were combined to prepare GD3-KLH and GD3-L-KLH conjugate vaccine (see Material and Methods).

80% of the GD3 had been lactonized, the acetic acid was quickly removed using a Centriprep (Amicon, Beverly, MA; 30 kDa molecular cut-off filter) with multiple saline washes. The conjugate was sterilized by passing through a 0.22-μm filter. The amount of GD3 or GD3-L in the conjugate was determined by estimating the sialic acid content by the resorcinol method (Svennerholm, 1963). GD3-KLH or GD3-L-KLH conjugate containing 30 μg ganglioside was aliquoted to individual vials and lyophilized under sterile conditions. In both cases, prior to the injection, 100 μg of QS-21 were mixed with the vaccine as it was reconstituted in normal saline solution.

Patients and clinical protocol

Patients with AJCC stage III or IV metastatic malignant melanoma (regional or systematic metastases) who were free of detectable melanoma within 2 weeks to 6 months after surgery were candidates for this trial. No patient had received prior chemotherapy. GD3-KLH or GD3-L-KLH conjugate containing 30 μg of ganglioside and 100 μg immunological adjuvant QS-21 were mixed immediately prior to vaccine administration in a total volume of 1 ml saline. Four vaccinations were administered s.c. at 1-week intervals, 2 additional vaccinations were administered at

TABLE II - PEAK ANTIBODY RESPONSE OF PATIENTS AFTER VACCINATION WITH GD3-KLH OR WITH GD3-L-KLH AS DETERMINED BY ELISA

Patient	Peak reciprocal ELISA titer							
	GD3				GD3-L			
	IgM		IgG		IgM		IgG	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
GD3-KLH lyophilized								
1	0	0	0	0	NT[1]	NT	NT	NT
2	0	0	0	0	NT	NT	NT	NT
3	20	80	0	80	NT	NT	NT	NT
4	0	0	0	0	NT	NT	NT	NT
5	0	0	0	0	NT	NT	NT	NT
6	0	20	0	20	NT	NT	NT	NT
GD3-L-KLH lyophilized								
1	0	40	0	160	0	40	0	160
2	0	0	0	0	0	40	0	0
3	0	40	0	20	0	160	0	0
4	0	160	10	1,280	0	640	0	1,280
5	0	40	0	160	0	40	0	160
6	0	1,280	0	320	0	1,280	0	1,280

[1]Not tested, because of the absence of antibodies against GD3 antibody.

intervals of 7 and 19 weeks from the date of first vaccination, under an MSKCC IRB approved protocol. Peripheral blood (20–30 ml) was drawn immediately before each vaccination, and 2 weeks after the 4th, 5th and 6th vaccinations.

ELISA

ELISAs were performed as described previously (Helling *et al.*, 1995). To determine the titers of GD3 and GD3-L antibodies, ELISA plates were coated with GD3-L or GD3 at 0.1 μg per well in ethanol. Serially diluted patient serum was added to wells of the coated plate and incubated for 1 hr at room temperature. Rabbit anti-human IgM or IgG conjugated with alkaline phosphatase served as second antibodies. The antibody titer was defined as the highest serum dilution showing an absorbance 0.1 or greater over that of normal sera. Immune sera were also tested for non-specific "stickiness" on plates that were processed identically but without ganglioside, and reading was subtracted from the value obtained in the presence of gangliosides.

Immune thin layer chromatography (ITLC)

Immune staining of gangliosides with MAbs or human sera was performed after separation of purified gangliosides or melanoma extracts on HPTLC silica gel glass plates as described previously (Hamilton *et al.*, 1993). The plates were coated with 1% Plexigum (Polyscience, Warrington, PA) in n-hexane, blocked with 3% BSA in PBS for 2 hr and incubated with patient sera (diluted 1:150 in PBS) overnight at room temperature. The plates were washed with PBS containing 0.05% Tween 20 (Fisher Scientific, Fair Lawn, NJ) and incubated with anti-human IgG or IgM antibodies conjugated with horseradish peroxidase at 1:200 dilution for 3 hr at room temperature. The plates were then washed with PBS-0.05% Tween 20 and developed with 4-chloro-1-napbtol-H_2O_2.

Dot-blot immune stain

Gangliosides GD3, GD3-L, GD2, GM1, GM2, GM3 and fucosyl (Fuc)-GM1 (0.1 μg) were spotted on nitrocellulose strips. The unreacted sites were blocked with 3% HSA-0.05% Tween-20 in PBS. The strips were treated as described for ITLC except coating with Plexigum. The intensity of spots in dot-blot immune stains was graded -, +, ++ or +++.

Fluorescence activated cell sorter (FACS) assay

The GD3 positive melanoma cell line SK-MEL-28 served as a target. Single cell suspensions of 2×10^5 cells per tube were washed with 3% FCS in PBS and incubated with 20 μl of 1:20 diluted antisera or MAb R24 for 30 min on ice. After washing the cells twice with 3% FCS in PBS, 20 μl of 1:15 rabbit anti-human IgG or IgM-labeled with FITC were added. The suspension was mixed, incubated for 30 min and washed. The percent positive population and mean fluorescence intensity of stained cells were analyzed using a FACS Scan (Becton-Dickinson, Mountain View, CA) (Zhang *et al.*, 1995).

Immune adherence (IA) assay

The IA assay measures rosetting of human RBC (blood group O) with guinea pig complement on target cells (SK-MEL-28) mediated by complement binding antibodies, and was performed as described previously (Shiku *et al.*, 1976). Individual target cells were scored as positive when 50% or more of the cell perimeter was surrounded by indicator cells.

Complement-dependent cytotoxicity (CDC)

CDC was assayed at a serum dilution of 1:10 with SK-MEL-28 cells and human complement by a chromium-release assay as previously described (Helling *et al.*, 1995). All assays were carried out in triplicate. Cells incubated only with culture medium, complement, antisera or MAb R24 served as controls. Spontaneous release was calculated based on the chromium released by target cells incubated with complement alone. Maximum release was determined by incubating target cells with complement and 1% Triton X-100. Percent cytolysis was calculated according to the formula:

Specific release (%)

$$= \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Inhibition assay

Antisera at 1:150 dilution or MAb R24 at 1 μg/ml were mixed with various concentrations of structurally related and unrelated ganglioside antigens. The mixture was incubated at room temperature for 30 min and tested on a GD3-coated plate by ELISA. Percentage inhibition was calculated as the difference in absorbance (ELISA) between the uninhibited and inhibited serum.

RESULTS

Clinical considerations

All patients signed informed consent prior to vaccination. Complete blood counts (CBC), liver function tests and clinical evaluations were repeated at 2-month intervals. Toxicity was restricted

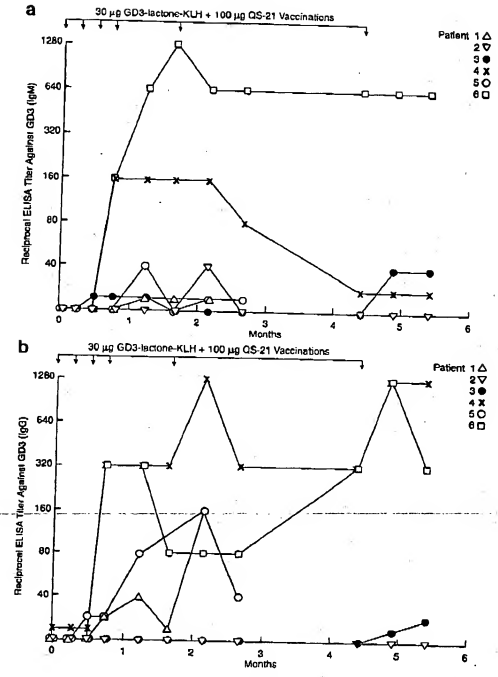

FIGURE 2 – Time course of the induction of IgM *(a)* and IgG *(b)* antibodies in 6 patients immunized with GD3-L-KLH plus QS-21.

to grade II local erythema and induration at vaccination sites lasting 3–5 days in all patients and grade 1 fever and flu-like symptom lasting 1–2 days after 2–3 immunizations in one half of the patients. This is the pattern of side effects associated with QS-21 administration at the 100-μg dose (Livingston *et al.*, 1994). No other side effects were detected.

ELISA responses against GD3 and GD3-L

The ELISA titers against GD3 and GD3-L are summarized in Table II. Before vaccination, IgG antibodies against GD3 or GD3-L were detected in 1 patient (titer 1/10) and IgM antibodies were detected at a titer of 1/20 in 1 patient. After vaccination, only one patient vaccinated with GD3-KLH developed detectable IgM and IgG antibodies against GD3 (titers 1/80). Consequently, further analysis was not carried out with GD3-KLH sera. The GD3-L-KLH vaccines, however, induced IgM titers against GD3-L of 1/40–1/1,280 in all patients and IgG titers of 1/160–1/1,280 in 4 patients (Table II). These antibodies also strongly cross-reacted with GD3. Peak reciprocal IgM titers of 40–1,280 were seen against GD3 in 5 of 6 patients and peak reciprocal IgG titers

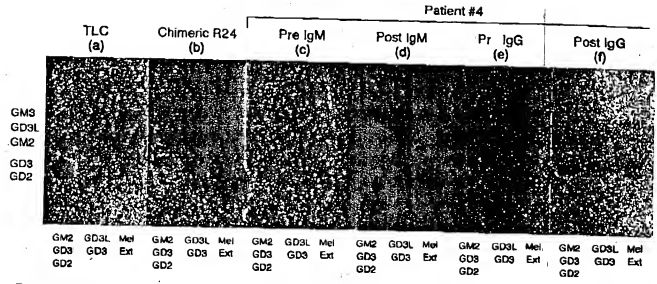

FIGURE 3 – ITLC with GD3, GD3L, GM2, GD2 and melanoma extract and sera from patient vaccinated with GD3-L-KLH. Chimeric MAb R24 was used as a control.

TABLE III - ANTIBODY RESPONSE OF PATIENTS AFTER VACCINATION WITH GD3-L-KLH AS DETERMINED BY DOT-BLOT[1]

Patient number		IgM							IgG						
		GD3-L	GD3	GD2	GM1	GM2	GM3	FGM1	GD3-L	GD3	GD2	GM1	GM2	GM3	FGM1
1	Pre	–	–	–	+	–	–	±	–	–	–	–	–	–	–
	Post	+++	+++	–	+	–	+	+	+++	+++	–	–	–	–	–
2	Pre	–	–	–	+	+	–	++	–	–	–	–	–	–	–
	Post	+++	+++	–	+++	+	+++	–	±	–	–	–	–	±	–
3	Pre	±	–	–	±	–	–	+	–	–	–	–	–	–	–
	Post	+++	+++	–	–	++	–	+	+	+	–	±	++	–	–
4	Pre	–	–	–	–	–	+	–	–	–	–	–	–	–	–
	Post	+++	+++	–	–	–	+	–	+++	++	–	–	–	–	–
5	Pre	–	–	–	–	±	–	–	–	–	–	–	–	–	–
	Post	+++	+++	–	±	–	+++	–	+++	++	–	–	–	–	–
6	Pre	–	–	–	–	–	–	++	–	–	–	–	–	–	–
	Post	+++	+++	+++	–	–	–	++	+++	+++	–	–	–	–	–
R24		±	–	–	–	+++	–	–	+++	+++	–	–	–	–	–
696															

[1]Dot-blot immune stains were graded: – (negative), + (weakly positive), ++ (positive) and +++ (strongly positive). FGM1, fucosyl GM1; R24 and 696 are MAbs against GD3 and GM2, respectively.

against GD3 were 160–1,280 in 4 of 6 patients. The ELISA time course of IgM and IgG antibody induction against GD3 with all sera in all 6 patients receiving the GD3-L-KLH vaccines is shown in Figure 2. IgM antibody titers remained higher than IgG titers at most time points, including after the 2 booster vaccinations. In general, titers were no higher after the booster vaccinations then after initial vaccinations.

Immune response with other gangliosides by ITLC and dot-blot

Pre- and post-vaccination sera of all 6 patients receiving the GD3-L-KLH vaccine were tested by ITLC for reactivity with GD3-L, GD3, GD2 and GM2 as well as with melanoma extract. The immune reactivities of pre- and post-vaccination sera from patient 4 along with chimeric MAb R24 are shown in Figure 3. Sera from 5 of 6 patients showed IgM antibody reactivity as strong as patient 4 against GD3-L and GD3. Sera from patients 1, 4, 5 and 6 also showed comperable IgG reactivity with GD3-L and GD3. Most of the IgM antibodies cross-reacted weakly with GM2 and GD2, whereas IgG antibodies showed only weak cross-reactivity with GD2. Both IgM and IgG reactivities were also seen with higher migrating bands in the melanoma extract, GD3-L.

The specificity of ganglioside antibodies detected in patient sera before and after immunization was also determined by dot-blot immune staining on nitrocellulose membranes containing gangliosides GD3-L, GD3, GD2, GM1, GM2, GM3 and Fuc-GM1. The results are summarized in Table III. A strong positive reactivity of 3+ for IgM antibodies against GD3-L and GD3 was seen in the sera of all 6 patients. Reactivity of 3+ for IgG antibodies was seen in 4 patients with GD3-L and GD3. Pre- immunizaton IgM and IgG antibodies from all patients showed no reactivity with GD3, although some patients had a low pre-immunization IgM antibody level against GM2 and GM1. Reactivity with these gangliosides

TABLE IV – CELL SURFACE REACTIVITY AGAINST SK-MEL-28 CELLS OF PATIENT SERA AFTER VACCINATION WITH GD3-L-KLH[1]

Patient	SK-MEL-28							
	FACS				IA		CDC	
	IgM		IgG					
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	9.5	9.7	11.0	35.5	Neg	Neg	4.3	7.0
2	8.9	9.7	10.7	19.8	Neg	Neg	1.5	3.0
3	5.5	13.6	9.9	23.4	Neg	1:5	1.9	2.3
4	10.5	8.7	9.8	95.8	Neg	1:10	1.0	56.0
5	5.0	4.8	2.3	10.5	Neg	Neg	3.4	4.5
6	9.1	80.2	11.5	36.2	Neg	1:40	5.4	51.9

[1]MAb R24 (IgG_3) showed 96.75% positive cells by FACS and 26.6% lysis on SK-Mel-21 cells.

was increased after vaccination in 1 patient each against GM1, Fuc-GM1, GM2 and GD2 and in 2 patients against GM3.

Reactivity of antisera with tumor cells

The cell surface reactivity of peak titer post-immunization antibodies was tested on GD3 positive SK-MEL-28 melanoma cells by FACS, IA and CDC assays. The results with sera from patients receiving the GD3-L-KLH vaccine are summarized in Table IV. The percent positive cells by FACS with pre-vaccination sera were low. Sera from patient 6 showed strongly increased IgM reactivity against SK-MEL-28 cells by FACS, and sera from 4 of 6 patients showed IgG reactivity of over 20% after vaccination. Sera from 3 patients showed post-immunization IA reactivity against SK-MEL-28, and sera from 2 patients showed more than 50% CDC against SK-MEL-28. Under similar conditions, specific release with MAb R24 was 26.6%. Post-vaccination sera in the absence of complement and complement without sera were not cytotoxic.

Antibody specificity determined by inhibition

Two different types of inhibition assays were carried out to define further the specificity of GD3 antibodies in immune sera: (1) incubation of sera with GD3, GD2, GM2 or GM1 and testing back against GD3 by ELISA; and (2) incubation of sera with GD3 and testing back against SK-MEL-28 by FACS. A sample experiment demonstrating the inhibition of IgM and IgG ELISA reactivity for patient 6 (who had shown IgM reactivity against GD2 by dot-blot and TLC) is shown in Figure 4. The results obtained from all 6 patients are summarized in Table V. The results indicate that GD3 inhibited anti-GD3 IgM at least 10-fold more efficiently than GD2, whereas GM2 and GM1 exhibited no inhibition. The anti-GD3 IgG antibodies were specific for GD3 alone; no other ganglioside inhibited GD3 reactivity.

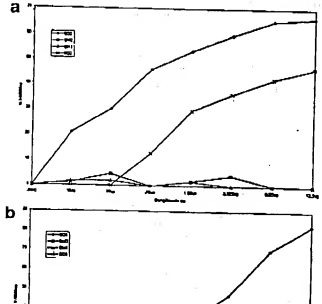

FIGURE 4 – Inhibition of ELISA reactivity of anti-GD3 IgM *(a)* and IgG *(b)* antibody with GD3, GD2, GM1 and GM2 gangliosides (patient 6).

DISCUSSION

Of the melanoma gangliosides considered to be potential targets for immunotherapy, GD3 is the most abundant but also the least immunogenic. Its potential as a target for passive immunotherapy has been documented in patients treated with R24, a murine MAb recognizing GD3. Regression of melanoma metastases after R24 treatment has been demonstrated at several different centers (reviewed by Jurcic *et al.*, 1997; Scheinberg and Chapman, 1995). We have spent considerable effort over the years trying to construct an effective vaccine against GD3 (Livingston, 1995; Ritter *et al.*, 1990b, 1991). Our initial approaches were to vaccinate melanoma patients with melanoma cells, whole cell lysates or with purified ganglioside adsorbed to BCG. Using these methods, we were able to induce antibody against GM2 but not against GD3 (Helling *et al.*, 1995; Livingston *et al.*, 1982; Ritter *et al.*, 1991). These experiences led us to search for ways to improve the poor immunogenicity of GD3. Several reports indicated that chemical modification of gangliosides could augment their immunogenicity. We prepared a series of synthetic ganglioside congeners and adhered them to BCG to induce antibody in laboratory animals and melanoma patients (Ritter *et al.*, 1990*a*,*b*, 1991). GD3 amide, GD3 ganglioasidol, GD3-L I and II and GD3 acetylated at various sites were all more immunogenic than GD3, but the increased antibody titers induced by these synthetic congeners of GD3 were not reactive with unmodified GD3 or melanoma cells (Ritter *et al.*, 1990*a*,*b*). Low-titer GD3 reactive autoantibodies have been reported in the serum of some melanoma patients after vaccination with irradiated melanoma cells (Ravindranath *et al.*, 1989), but less frequently and far lower titer than antibodies against GM2 and GD2, and no cell surface reactivity could be demonstrated. However, human MAbs reactive with GD3 have been generated (Yamaguchi *et al.*, 1987). It was clear that GD3 was a poor immunogen in humans, but never the less could be recognized by the human immune system.

Immunization of mice with GM3-L induced antibodies that cross-reacted with unmodified GM3 (Nores *et al.*, 1987) and a

TABLE V – INHIBITION OF GD3 ANTIBODY ACTIVITY WITH DIFFERENT GANGLIOSIDES[1]

Sera	GD3 target				
	IgM		IgG		
	GD3	GD2	GM2	GD3	GM2
Patient 1*	3+	2+	2+	NT[2]	NT
Patient 2*	1+	1+	0	NT	NT
Patient 3	1+	0	0	NT	NT
Patient 4	4+	1+	0	NT	NT
Patient 5*	3+	0	0	4+	0
Patient 6	4+	2+	0	NT	NT
			4+	0	0

[1]Grading scale: percent inhibition of ≥85% was given a grade of 4+; 70%–85% 3+; 40%–70% 2+; 20%–40% 1+; below 20% 0. GM1 did not inhibit any sera.–[2]NT: not tested.–*Inhibiting on sera with ≥1/40.

murine MAb reactive with both GM3 and GM3-L has been generated (Dohi *et al.*, 1988). Murine MAbs reactive with other ganglioside lactones are also reactive with the parent ganglioside (Bosslet *et al.*, 1989; Dohi *et al.*, 1988; Tai *et al.*, 1988). Serum antibodies induced by immunization of mice with GD3-LI (the lactone ring formed between the carboxyl group of sialic acid and the hydroxyl group of the ganglioside) were shown to react with purified GD3 and GD3 expressing human melanoma cells (Ritter *et al.*, 1990a). With GD3-L/BCG, we were able to induce low- titer antibodies against GD3-L in 4 of 9 patients. However, these antibodies were exclusively IgM, the response was of short duration and no reactivity against GD3 was seen. These results suggested that GD3-L/BCG was a more potent method of immunizing against GD3 than GD2/BCG, but was not potent enough (Ritter *et al.*, 1991).

Several other approaches have been reported to augment the immunogenicity of carbohydrate antigens (Helling *et al.*, 1995; Ragupathi *et al.*, 1998). Covalent attachment of carbohydrate antigens to immunogenic protein carriers as first suggested for haptens and then carbohydrates is the concept that has been pursued most vigorously, especially in vaccines against infectious diseases. Regarding conjugate vaccines against gangliosides, in our initial studies with GD3 vaccines in the mouse, we established the optimal conjugation method, the optimal carrier protein and the necessity for a potent adjuvant (Helling *et al.*, 1994). The optimal conjugation procedure for GD3 was cleavage of the ceramide double bond with ozone, generation of an aldehyde group and coupling to free ε-amino groups of the lysine of protein by reductive amination. We found that KLH was the optimal carrier and QS-21, a homogeneous saponin fraction purified from the bark of *Quillaja saponaria*, the most effective adjuvant (Helling *et al.*, 1994). Mice vaccinated with GD3-KLH conjugate plus QS-21 had higher titer IgM antibodies and consistent production of high IgG antibody titers. The superior immunogenicity of the KLH conjugate vaccine plus QS-21 has also been demonstrated in melanoma patients with GM2-KLH (Helling *et al.*, 1995).

Putting together the increased immunogenicity of KLH conjugate plus QS-21 vaccines, the basic ability of the human immune system to produce antibodies recognizing GD3 and the increased immunogenicity of ganglioside GM3-L compared to GM3 in terms of anti-GM3 antibodies, it seemed reasonable to reevaluate the immunogenicity of GD3 and GD3-L using KLH conjugate plus QS-21 vaccines. Others have described clinical trials that occasionally induced antibodies against GD3 in patients (Portoukalian *et al.*, 1991; Ravindranath *et al.*, 1988). We report here an immunization procedure that resulted in the production of IgM and IgG antibodies against GD3 and tumor cells expressing GD3 in the majority of immunized patients. The antibodies produced in response to immunization with GD3-L were specific for GD3-L but also cross-reacted significantly with purified melanoma GD3, OD3 isolated from bovine buttermilk and GD3 on the melanoma cell surface. This is in contrast to the experience of others with MAbs raised against GD3-L which reacted with GD3-L but not with GD3 (Kawashima *et al.*, 1993, 1994). GD3-KLH failed to induce antibody against GD3, suggesting that tolerance to GD3 as a consequence of expression of GD3 on a variety of normal human tissues could be broken by GD3-L but not by GD3. This may be because GD3-L is expressed at lower levels on normal tissues and so is more easily recognized by the immune system, or because GD3-L is a more rigid, less flexible molecule that consequently is a stronger immunogen.

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